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Quantitative analysis of germline mitosis in adult *C. elegans*

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Abstract

Certain aspects of the distal gonad of *C. elegans* are comparable to niche/stem cell systems in other organisms. The distal tip cell (DTC) caps a blind-ended tube; only the distal germ cells maintain proliferation in response to signaling from the DTC via the GLP-1/Notch signaling pathway in the germ line. Fruitful comparison between this system and other stem cell systems is limited by a lack of basic information regarding germ cell division behavior in *C. elegans*. Here, we explore the spatial pattern of cell division frequency in the adult *C. elegans* germ line relative to distance from the distal tip. We mapped the positions of actively dividing germline nuclei in over 600 fixed gonad preparations including the wild type and a gain-of-function ligand-responsive GLP-1 receptor mutant with an extended mitotic zone. One particularly surprising observation from these data is that the frequency of cell divisions is lower in distal-most cells—cells that directly contact the distal tip cell body—relative to cells further proximal, a difference that persists in the gain-of-function GLP-1 mutant. These results suggest that cell division frequency in the distal-most cells may be suppressed or otherwise controlled in a complex manner. Further, our data suggest that the presence of an active cell division influences the probability of observing simultaneous cell divisions in the same gonad arm, and that simultaneous divisions tend to cluster spatially. We speculate that this system behaves similarly to niche/stem cell/transit amplifying cell systems in other organisms.

Keywords: *C. elegans*; Germ line; Mitosis; Stem cell; Intensity profile; Spatial statistics; GLP-1

Introduction

The behavior and control of continuously dividing, anatomically restricted cell populations are areas of intense research. Recent studies indicate that many tissues are maintained by stem cells that are capable of dividing throughout the lifetime of an organism. Stem cells from a variety of adult tissues reside in anatomically distinct locations adjacent to a "niche" or microenvironment that maintains stem cell fate (see Fuchs et al., 2004; Li and Xie, 2005; Ohlstein et al., 2004; Spradling et al., 2001; Watt and Hogan, 2000 for reviews). The distal tip cell (DTC) of the *C. elegans* gonad is comparable to a niche in that it is responsible for maintaining the nearby germ cells in a mitotic (undifferentiated) state as opposed to a meiotic (differentiated) state (Kimble and White, 1981). Germline stem cells, however, have not been directly identified in this system. An understanding of the pattern of germ cell divisions adjacent to the DTC will facilitate comparison with other systems. However, informative measurements of cell division behavior have been difficult to obtain. Some of this difficulty is due to system-specific technological and reagent limitations that can be overcome with future improvements. Much of the difficulty, however, is due to inherent features of the system such as the low frequency and variable spatial distribution of observable cell division events. Some of these difficulties can be surmounted by quantitative analysis.

The *C. elegans* germ line contains an anatomically restricted mitotic cell population that persists throughout the life of the worm and, based on the reproductive capacity of the organism, is thought to be self-renewing. In contrast to the invariant and

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fully described cell lineage of the *C. elegans* soma (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston et al., 1983), germ cell divisions are variable with respect to order and division plane (Kimble and Hirsh, 1979). The early adult hermaphrodite germline mitotic zone is located in the distal-most end of the gonad arm and contains roughly 250 nuclei in various stages of the mitotic cell cycle (Fig. 1). Proximal to this zone, cells enter the meiotic pathway and eventually either give rise to gametes or undergo programmed cell death (Gumienny et al., 1999; Hirsh et al., 1976).

The mitotic (as opposed to meiotic) state of nuclei in the *C. elegans* germline distal region is maintained by interaction with the distal tip cell (DTC) (Kimble and White, 1981) via a canonical Notch signal transduction pathway mediated by the GLP-1 receptor (see Crittenden et al., 2003; Kimble and Crittenden, 2005; Westlund et al., 1997 for reviews). In the absence of the DTC (or signaling pathway components), all germ cells differentiate and the mitotic zone is lost (Austin and Kimble, 1987; Henderson et al., 1994; Kimble and White, 1981; Lambie and Kimble, 1991; Tax et al., 1994; Yochem and Greenwald, 1989). Moreover, increased activity of the GLP-1 receptor leads to germline tumor formation (Berry et al., 1997; Pepper et al., 2003a). Although the activity of this particular Notch pathway has not been directly tied to the cell cycle, as it has in other organisms (e.g., Sarmento et al., 2005), GLP-1-mediated signaling opposes the activities of at least three partially redundant pathways (Eckmann et al., 2004; Hansen et al., 2004a,b; Kadyk and Kimble, 1998; Lamont et al., 2004) that are important to position the mitosis/meiosis border relative to the distal tip.

Despite the promise this anatomically simple and experimentally tractable system holds for investigation of adult dividing cell populations, many key features of the system remain poorly defined. Active divisions in the adult mitotic zone are too infrequent to analyze in live specimens or in small numbers of fixed preparations (although the latter are sufficient to analyze steady-state levels of proteins or RNAs). Furthermore, lineage-tracing and transplantation approaches are not yet technically feasible, and cell-specific markers for stem cells versus non-stem mitotic cells are not available. Ablation of one of the two founder germ cells delays but does not otherwise impair repopulation of the gonad (Kimble and White, 1981), and systematic germ cell ablations have not been reported. Thus, putative stem cells have not been

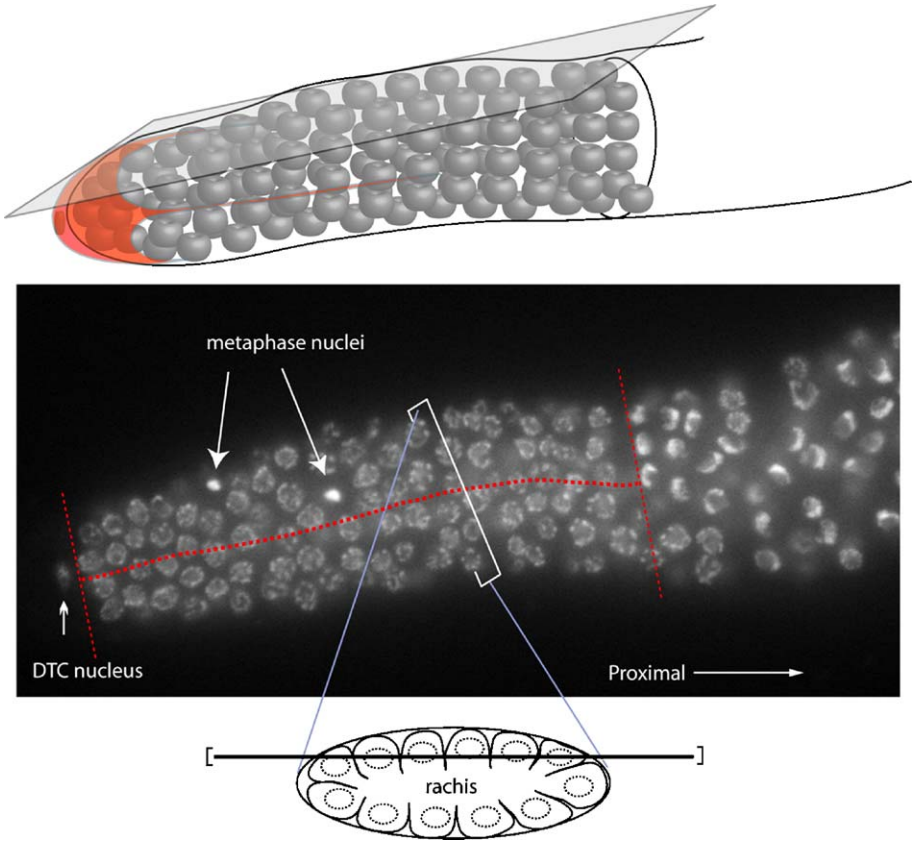

Fig. 1. The distal end of an adult hermaphrodite gonad arm. (Top) Cartoon representation of distal mitotic zone of adult *C. elegans* hermaphrodite with an optical section represented as a plane. Germline nuclei are grey, the DTC is red. The DTC, though seen in transverse section as a crescent capping the germ line, actually cups the germ cells close to the distal tip. (Middle) Photomicrograph of one optical section (surface section) through a fixed, DAPI-stained preparation. Nuclei in metaphase of mitosis are indicated by two arrows. Small arrow points to the DTC nucleus. Red dotted lines indicate the borders of the mitotic zone and the "vein" (see Materials and methods). (Bottom) Cartoon of cross-section of a fixed, mounted gonad arm indicating the approximate focal plane of the image above. Germ nuclei are depicted as dashed-line circles. Germ "cells" – each nucleus and its surrounding cytoplasm – retain an opening to a core of shared cytoplasm (the rachis) in a part of the cell membrane surface that faces the rachis (because the cartoon represents one plane, the actual size of the opening to the rachis appears exaggerated on most cells).

identified, the dynamics of cell division within the zone are unknown, and the lineal relationships between cells in the zone are not defined.

Finally, the relationship between the frequency of cell division and distance from the DTC position is unclear. One possible expectation is that the frequency of divisions in the distal zone is constant across the distal half of the mitotic zone where membrane-associated GLP-1 protein levels are high and apparently constant, suggesting that the cells in the distal half of the zone are equivalent. An alternative possibility is that the probability of observing a cell division may differ depending on distance from the distal tip, suggesting that distal germ cells are not equivalent. For example, distal-most cells—those contacting the DTC body—may display a frequency of division that differs from cells more proximal in the zone.

We investigated the frequency with which cell divisions occur within the mitotic zone as a function of distance from the DTC and from each other using a quantitative approach. We applied spatial statistics to analyze cell division events from a large number of fixed preparations. Our analysis provides three important advances in our understanding of cell division behavior within the distal mitotic zone. First, we define the frequency of divisions as a function of distance from the distal tip in the wild type and in a mutant with elevated GLP-1 activity. These data enable us to reject several hypotheses concerning the behavior of cells in the proliferation zone. In particular, we find that the distal-most cells, corresponding to those in contact with the DTC body (Hall et al., 1999; Fig. 1), display a lower frequency of division than adjacent cells. Second, we find that active divisions per adult gonad arm are infrequent and do not fit a Poisson distribution, suggesting that the probability of observing simultaneous divisions is influenced by the presence of other divisions in the same gonad arm. Third, we find that synchronous divisions are not distributed randomly with respect to each other and, rather, tend to cluster. These studies provide a baseline for further comparisons under other life-stage and genetic conditions, and demonstrate the utility of a quantitative approach to the analysis of any anatomically restricted dividing cell population. Based on our findings, we propose a model for cell division behavior in the *C. elegans* adult germline mitotic zone and draw comparisons with niche/stem cell systems in other organisms.

Materials and methods

Worm handling

All strains were manipulated using standard methods. Synchronization, fixation, and staining (see Supplementary material) were carried out essentially as described (Pepper et al., 2003a,b). Animals growing at 20° were synchronized as early L1 larvae by hatch-off, subsequently reared at 25°, and harvested for analysis 48 h later.

Image acquisition and manual analysis

Images (1 μm Z-stacks) were collected on a Zeiss Axioplan2 microscope using a Hamamazu Orca camera, and a PowerMac G4 running Improvision Openlab version 3.5.0. For the manual mitotic index analysis (Fig. 2A), the cells in each "cell diameter" interval (CD) were counted and the locations and number of actively dividing nuclei were noted. For the computer-assisted analysis, the mitotic zone (the area between the distal tip and the first transition nucleus) and the locations of individual mitotic figures (x, y, and z coordinates) were marked (Fig. 1; see Supplementary material). The distal mitotic zone was defined as the distal-most point of the distal-most germ cell to the first transition nucleus. We refer to distance from the distal end in three different measures: pixels, microns, and "cell diameters". See Supplementary material for additional details.

Data analysis

Raw image data were processed to enable consistent statistical analysis. The processing computationally "straightened" gonad arms that were bent in the xy plane, while maintaining local geometry and spatial distributions (Supplementary material).

Analysis of position of observed divisions per arm; the intensity measure

Although our data specify the location of each division in 3-dimensional space, here we consider only the x-coordinate position of the divisions: the distance of a division from the distal tip measured along the vein (see Fig. 1; Supplementary material). The full 3-dimensional analysis will appear elsewhere. We model the data as a point process; we analyze data using a non-parametric approach that, due to sample size, we limited to first and second order statistics (i.e., analyzing the means in every location and covariances of the point process distribution).

The intensity profile

For any point process, the intensity function is a positive-valued function, defined such that the expected number of points in any interval is the integral over that interval of the intensity. We estimated the intensity non-parametrically through kernel estimation (Silverman, 1986), correcting for two effects: tapering of the arm at the distal end and boundary effects (Fig. 2; Supplementary material). These corrections did not substantially alter the intensity profile, further indicating the robustness of this measure. Confidence intervals (95%) for the intensity function were calculated using an adaptation of previously described methods (Bickel and Rosenblatt, 1973; Hardle, 1991; see Supplementary material for details).

Analysis of spatial clustering/repulsion among divisions

To determine if locations of divisions are interdependent within the same gonad arm, we first made a coordinate change by applying an inverse function transformation. This is a standard technique that allows the reduction of the study of a 1-dimensional point process with known intensity to one with constant intensity. The point process was then analyzed by estimating Ripley's K-function (Dixon, 2002) and $K'(x)$, the derivative of K (Fig. 3; Reilly et al., 2002; Supplementary material).

Results

The DTC is the source of a ligand for the GLP-1/Notch signaling pathway that promotes/maintains germ cell mitosis (and/or inhibits meiosis). The activity of this pathway is therefore responsible for the distal–proximal polarity of the germ line. The level of activity of this pathway also correlates with the size of the proliferation zone (Austin and Kimble, 1987; Berry et al., 1997; Crittenden et al., 1994; Pepper et al., 2003a). It is not evident, however, how the polarity and/or GLP-1 pathway activity are related to the frequency of cell divisions within the zone. We therefore examined the spatial distribution of cell division events as a function of the distal–proximal axis.

To determine whether cell division frequencies are similar over the distal half of the mitotic zone, a region where levels of membrane-associated GLP-1 protein appear uniformly high (Crittenden et al., 1994), we calculated a mitotic index by "cell diameter" intervals (CD) from the distal tip (Fig. 2A). For this and all subsequent analysis, we used worms synchronized at an early adult time-point (1) that corresponds to the peak size of the hermaphrodite mitotic zone (Killian and Hubbard, 2005), (2) in which all mitotic germ cells possess a uniform oogenic fate (as opposed to earlier time points in which mitotic zone cells may be destined to form sperm) (Ellis and Schedl, in press; Hirsh et al., 1976), (3) when a large supply of sperm is present ensuring relatively uniform efflux of germ cells from the system by ovulation (McCarter et al., 1999), and (4) when the DTC is the sole proliferation-promoting influence on the mitotic zone (Killian and Hubbard, 2005; Kimble and White, 1981; Pepper et al., 2003b). At this time-point, the distal mitotic zone (distal end to the first transition nucleus) comprises 236 ± 27 germline nuclei (n = 35 gonad arms). We gathered cell division and cell count data from 3-dimensional "snapshots" of germline proliferation (Z-series images at 1 μm intervals; Materials and methods; Supplementary material).

To generate a mitotic index for the first 10 CDs, we counted the total number (total in all 50 gonad arms) of actively dividing nuclei in each CD (metaphase and anaphase/telophase; see Supplementary material for additional information regarding scoring criteria) and divided by the total number of nuclei in that CD (Materials and methods; Fig. 2A;

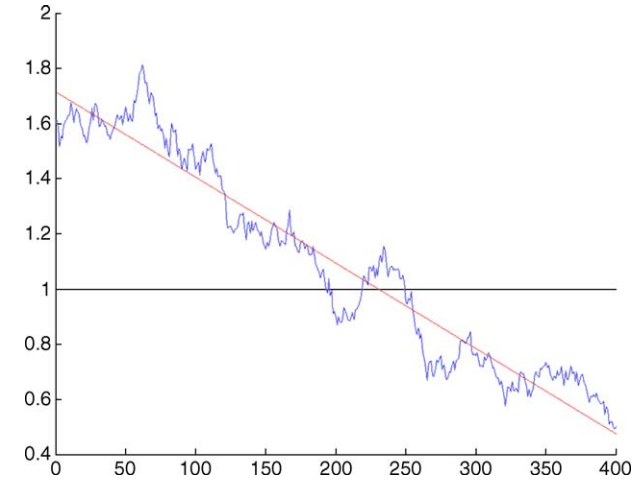

Fig. 3. $K'(x)$, the derivative of Ripley's K-function for the adult wild-type data set. To generate this plot, the wild-type intensity with bandwidth 33 was used with an averaging range of 30 pixels for smoothing. The x axis is distance (in pixels) between simultaneous divisions, and the y axis is $K'(x)$. Blue is the derivative of K, red is the linear fitting, and black is the horizontal line at $y = 1$ to indicate the expected outcome in the case of complete spatial randomness (i.e., a Poisson process). See Supplementary material for additional details.

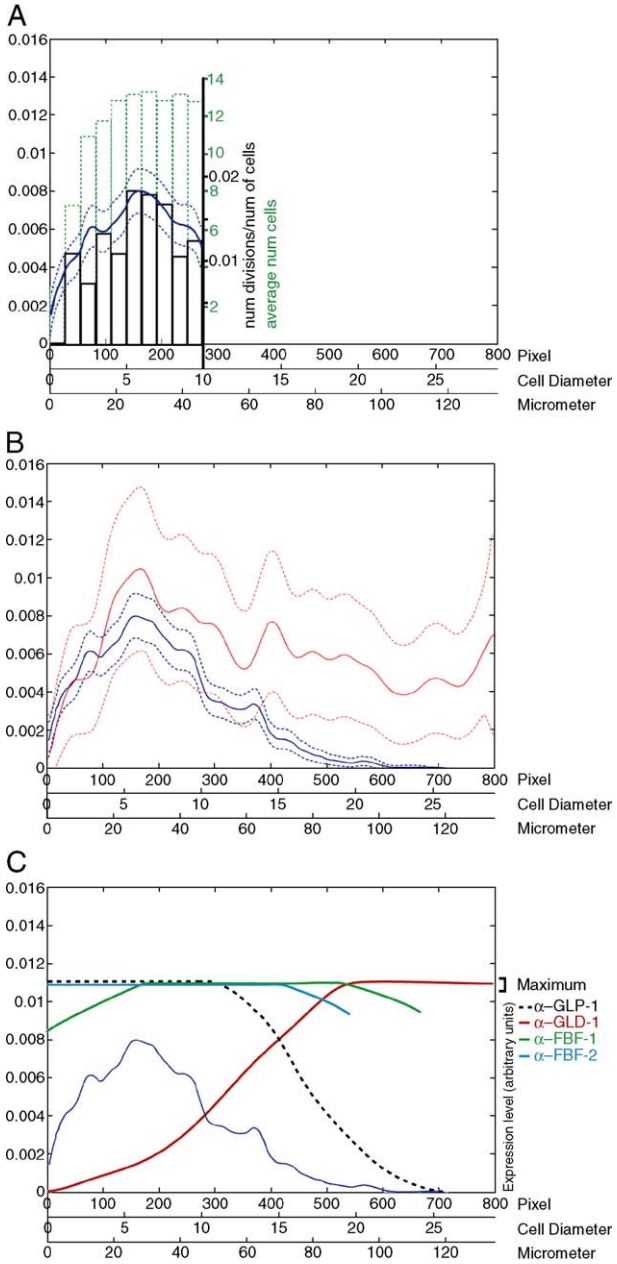

Fig. 2. Histogram of mitotic index and intensity profiles. (A) Histograms depicting mitotic index (black boxes; black right-hand y axis) and average number of cells (green boxes; green right-hand y axis) for each of the first 10 cell-diameter intervals from the distal end. The actual numbers of total (n = 50 gonad arms) cell divisions/cell number per CD (1–10) are 0/188, 4/367, 4/545, 8/592, 7/644, 12/660, 12/667, 11/645, 7/660, and 8/643. The left-hand y axis is the intensity (see Materials and methods; Supplementary material). The x axis is distance from the distal tip on three scales relating pixels, microns, and cell diameters (see Supplementary material). The intensity function with 95% confidence intervals (dashed lines) for (A) the wild-type adult (n = 587 gonad arms, kernel bandwidth = 33 pixels), and (B) wild-type (blue) plus the selected *glp-1(ar202)* mutants (red, n = 95 arms, bandwidth = 46 pixels; see Supplementary material and text for details). Though this data set can be used to compare to the wild type, we note that the confidence interval is quite large (dotted red lines). (C) The wild-type intensity profile from panel A overlaid with the approximate steady-state protein concentration levels estimated from previous publications (Crittenden et al., 1994; Hansen et al., 2004b; Jones et al., 1996; Lamont et al., 2004). For GLP-1, we depict the region of high penetrance of high-level cell membrane-associated protein only; significant levels of internal GLP-1 are present in the germ line proximal to CD 12 (see Crittenden et al., 1994, Figs. 4–6 for details). Levels are arbitrary between a maximum for each protein (indicated on graph) and a minimum (where $y = 0$).

Supplementary material). To our surprise, we observed no divisions (among 188 total nuclei in 50 gonad arms) in the first CD and only 1% dividing in CDs 2–5, whereas we observed an average of 1.8% in CDs 6–8. These data indicate that, on a per cell basis, the probability of observing an active division is lowest in the distal-most region and steeply rises to a peak by ~6 cell diameters from the distal tip.

The frequency of cell divisions as a function of distance from the distal tip: a complex function

Although the results from the analysis of 50 gonad arms were suggestive, given the generally low frequency with which we observed active divisions, we performed a larger-scale and more rigorous analysis to determine more precisely the cell division parameters in the entire distal zone. We also reconsidered our methods for data analysis. In addition to the laborious nature of the mitotic index data collection, there are drawbacks to the histogram approach (either histograms of cell division counts or of mitotic index; see Supplementary material). For example, the size and location of the bins are arbitrary, information about the exact location of divisions within each bin is lost, and further analysis of the spatial relationship between divisions within the same arm is not feasible. We therefore turned to spatial statistics methods for data analysis (Materials and methods; Supplementary material). For this analysis, we used Z-series images from 587 fixed age-synchronized wild-type gonad arms, manually marking the locations of the distal end of the germ line and all visible mitotic figures (see Materials and methods). Our analysis methods included a correction for changes in the size of the gonad arm across the zone.

From our analysis of 1295 divisions in the wild type, we computed a statistically robust intensity profile, a measure of the probability of finding a dividing cell at a particular distance from the distal tip (Figs. 2A, B). The area under the entire curve is the observed total average number of cell divisions per gonad arm (2.2). Similarly, the area under the curve for any x axis interval is equal to the average expected number of cell divisions within that interval in a single gonad arm. For example, reading from the intensity plot (Figs. 2A, B), the expected number of divisions for interval from 0–280 pixels (10 CD) is roughly 0.006 * 280 or 1.68. The expected number of divisions for interval from 150–200 pixels (~6–7 cell diameters) is ~0.0078 * 50 pixels or 0.39 divisions (that is, on average, 39/100 gonads will have a division occurring in this interval). These values agree well with the manual data collected from 50 gonad arms for which the average number of cell divisions per arm was 1.46 in the entire 10 CD interval and 0.48 in the CD 6 plus CD 7 interval. The intensity plot generated from the large data set is statistically rigorous: dotted lines above and below the intensity profile indicate 95% confidence intervals for this function (see Supplementary material).

As in the manual cell-count data, the most striking feature of the intensity profile for the distal half of the zone is that the distal-most region exhibits the lowest frequency of cell division, while the peak frequency of cell divisions occurred at a position of ~6–7 cell-diameters away from the distal tip (see Supplementary material). Cell divisions among the distal-most 2–3 cell diameters are observed less than half as frequently as at the peak position. In the proximal part of the zone, cells eventually enter a relatively long pre-meiotic S phase (Alberts et al., 2002) without subsequent mitosis. This process is likely reflected in the steady decrease in the observed frequency of cell divisions in this part of the zone, with the exception of a sub-peak at ~13–14 cell diameters (Fig. 2B). Thus, the intensity profile follows almost exactly the data collected by manually counting cells in each CD interval, clearly indicating a low frequency of observed cell divisions in the distal-most part of the zone.

Elevated glp-1 activity changes cell division behavior

To examine the correlation between division frequency pattern and *glp-1* activity, we collected similar data from the distal region of a mutant with elevated *glp-1* activity that displays an extended adult distal mitotic zone. An enlarged adult zone could result from several different mechanisms that are not mutually exclusive. One is a larger founder population of mitotic germ cells, another is an accelerated cell cycle or the reduction in time of quiescence, and a third is a change in the dynamics of cell movement in the zone. Any of these effects could be limited to specific sub-zones or could be uniformly increased. The intensity profile allows us to examine differences in the spatial nature of these effects.

We chose *glp-1(ar202)* for this analysis: previous analysis of this mutant demonstrated that the size of the mitotic zone in *glp-1(ar202)* mutants is comparable to the wild type at the time it is established in the L3 stage (thus eliminating one variable), and under certain temperature conditions, the distal zone expands (Pepper et al., 2003b). GLP-1 activity is elevated in this mutant and can bypass loss of the LAG-2 ligand, but genetic analysis also indicated that this receptor can still respond to the ligand when the ligand is present (Pepper et al., 2003b). We examined data from 95 gonad arms in which the proliferation zone had expanded beyond the normal limit to ≥30 cell diameters. We processed the data as for the wild type including only those divisions that occurred between 0 and 800 pixels (Table 1; Fig. 2B), since this distance is greater than the distance at which divisions are ever seen in the wild-type.

The wild-type and *glp-1(ar202)* intensity profiles bear important similarities and differences (Fig. 2B). Most notably, the mutant and wild-type intensity profiles overlap over the distal-most five cell diameters, after which the mutant diverges with a higher peak and subsequently maintains a higher frequency of division. The increase in peak amplitude could correspond to ligand-dependent activity while the overall increase could correspond to the ligand-independent activity of the receptor, consistent with previous genetic analysis (Pepper et al., 2003b). Our data suggest that the (unknown) mechanism that imposes a lower division frequency on the distal-most germ cells is present in the mutant as well as the wild type. A sub-peak at ~15 diameters appears in

Table 1
Frequency of observed cell divisions in wild-type and *glp-1(ar202)* distal mitotic zones

Simultaneous divisions/arm	Wild-type adult		*glp-1(ar202)* adult[a]	
	Gonad arms (#)	% of total arms	Gonad arms (#)	% of total arms
0	140	24	4	4
1	111	19	6	6
2	118	20	9	9
3	92	16	11	11
4	50	9	20	20
5	28	5	10	10
6	25	4	12	12
7	11	2	7	7
8	6	1	4	4
9	3	1	4	4
10	3	1	3	3
11	0	0	2	2
12	0	0	2	2
13	0	0	1	1
n	587		95	
Mean divisions/arm	2.206		4.895	
Variance	4.1298		8.3931	
Total divisions	1295		465	
Average zone cell count	236[b]		287[b]	
Overall mitotic index	0.008[b]		0.022[b]	

[a] Counts from 0–800 pixels (see text). Parental animals were continuously raised at 20° and their progeny were shifted to 25° as L1 larvae to produce a large majority with an extended distal mitotic zone by 48 h of subsequent development.

[b] Mitotic index was calculated on a subset of gonad arms for the wild-type adult and *glp-1(ar202)* adult as follows: wild-type adult $n = 28$, cell divisions/arm = 1.9, *glp-1(ar202)* adult, $n = 30$ with average cell count (to 800 pixels), and cell divisions/arm = 6.3.

the mutant, very close to a similar peak in the wild type. This sub-peak could correspond to a second wave of division of the daughter cells generated in the first peak at ~6 cell diameters. Thus, elevated *glp-1* activity does not compensate for the lower cell division frequency among the distal-most germ cells and, rather, increases the cell division frequency of cells that are outside the area of direct contact with the distal tip cell body.

Observation of active M phase is rare in wild type and is not Poisson distributed

From counts of synchronous cell divisions (Table 1), we draw several conclusions. First, the average probability of observing a division among the ~236 cells in the wild-type zone is low (2.2 divisions per arm). Thus, our methods capture less than 1/100th of the average cell cycle time. Second, the cell division counts do not fit a Poisson distribution (chi-square, $P < 0.001$; Table 1) neither for the adult wild type nor the mutant (mean of 5.0 and variance of 9.6, $P < 0.001$; Table 1). We find that, relative to a Poisson distribution with the same mean, the 0-division class and many-division classes are particularly elevated in the wild-type adult. These results suggest that the presence of a division within a gonad arm alters the conditional probability of observing a simultaneous division in the same arm. In other words, the divisions are temporally clustered.

Synchronously dividing cells cluster within a gonad arm

We next examined the simultaneously dividing cells in the wild type as a function of their distance from each other. We again considered divisions along the distal–proximal axis, taking into account the frequencies indicated by the intensity profile (see Materials and methods). If spatially regulated phenomena affect the frequency of observing simultaneous cell divisions within a gonad arm, some evidence of spatial clustering or repulsion of divisions within a single gonad arm should be evident. We computed the empirical Ripley's *K*-function $K(x)$ for the data from all 587 gonad arms (Dixon, 2002) and the derivative $K'(x)$ (Reilly et al., 2002) (see Materials and methods). $K(x)$ would measure the expected number of division events at a distance less than or equal to x from a given division (where x is ≤400 pixels; see Supplementary material). In the case of complete spatial randomness (i.e., a Poisson process), its derivative $K'(x)$ is a horizontal line at height 1. If clustering occurs on a particular distance scale x, $K'(x)$ will be greater than 1 around x while repulsion is indicated by $K'(x)$ less than 1. Our results indicate that cell divisions tend to cluster at short distances between cell divisions in a manner that is significantly different from a random distribution expected by a Poisson process (Fig. 3; Supplementary material).

Discussion

We have examined the pattern of active cell divisions in the adult *C. elegans* germ line. Despite the current lack of molecular markers, we are able to draw several interesting conclusions from a straightforward quantitative analysis. We have determined that cell divisions are, overall, infrequent and appear to be temporally and spatially clustered. We also found that the frequency of observing cell divisions is non-uniform over the mitotic zone, with a markedly low frequency observed among cells in the distal-most part of the zone. In addition, we found that the peak of observed cell division frequency coincides with a region where germ cells do not contact the distal tip cell body, and we observed an expected decrease in division frequency in the proximal part of the zone as cells enter pre-meiotic S phase. In a gain-of-function GLP-1 receptor mutant where the zone is extended, division frequency in the distal-most region is still reduced while the overall division frequency among more proximal cells is elevated.

Temporal and spatial coordination of cell divisions

Our observation of non-Poisson behavior of cell division counts in the adult mitotic zone counters the simplest hypothesis that simultaneous divisions are independent of each other within each gonad arm. Our additional observation of spatial clustering of divisions at short distances (given our

measure of intensity) could be the result of cell–cell communication or a temporal retention of cell cycle timing. In the first scenario, signals emanating from a dividing cell would positively influence the probability that a nearby cell will divide at the same time. In the second scenario, the history of divisions could be roughly maintained such that two cells born at the same time would retain a relatively fixed cell cycle time and thus would retain some degree of synchrony in their subsequent cell cycles. A third, and not necessarily mutually exclusive, possibility is that individual cells in a given region may be exposed to different LAG-2 levels. This could be especially important in areas proximal to the DTC cell body where adjacent cells may differ substantially in their proximity to a DTC extension carrying LAG-2. Likewise, cells in contact with the same DTC extension may behave similarly with respect to their cell cycle control. Further studies are required to distinguish these possibilities.

Relationship between known protein gradients in the distal zone and the pattern of cell division frequency

Our observation of non-uniform cell division frequency over the distal–proximal axis can be compared to known gradients of proteins that have been associated with control of the size of the distal zone and for which protein concentrations are non-uniform within the zone. Fig. 2C is a summary of the published expression patterns of four such proteins: GLP-1, GLD-1, FBF-1, and FBF-2 (Crittenden et al., 1994; Hansen et al., 2004b; Jones et al., 1996; Lamont et al., 2004). In short, the shape of the wild-type intensity profile is not recapitulated by any one of these protein concentration patterns (Fig. 2C). The decline in intensity in the proximal part of the zone parallels, but occurs slightly more distal to, the decline in high steady-state membrane-associated GLP-1 levels. The intensity does not correlate with uniformly high steady-state membrane-bound GLP-1 levels in the distal part of the zone: the intensity increases, peaks, and declines in the region where membrane-associated GLP-1 levels appear constant. Neither observation precludes the possibility that GLP-1 *activity* levels correlate more closely to the observed increase in the pattern of cell division frequency in the distal-most region. This correlation is of interest and can be more accurately examined once a reagent is available to directly measure the activity of GLP-1.

Two highly related proteins, FBF-1 and FBF-2, are also present in the distal mitotic zone where they act redundantly to maintain the mitotic zone in the late larva and adult stages (Crittenden et al., 2002; Lamont et al., 2004; Fig. 2C). In a complex mutant background that eliminates GLD-1, GLD-2, and FBF-1, the expression of FBF-2 peaks at about 5 cell diameters from the DTC and then falls (Lamont et al., 2004) coincident with the increase and decrease in intensity we observe over the distal half of the zone in the wild type. Despite this tantalizing correlation, however, this complex genotype makes it difficult to draw a conclusion. In summary, our measurements of cell division frequencies as a function of distal-to-proximal distance cannot be easily reconciled to any single available protein level measurement, and likely relate to a complex interaction of the activities of these and additional proteins.

The intensity profile and cell division dynamics

Several general hypotheses can be envisaged that relate cell-cycle behavior to a response to GLP-1 pathway activity. One hypothesis is that the LAG-2-laden cell processes extending from the DTC (Fitzgerald and Greenwald, 1995; Hall et al., 1999; Henderson et al., 1994) influence the mitotic behavior of germ cells they contact. The region with the most density of processes, the first 8 cell diameters in early adults (Hall et al., 1999), does correlate to the area where the vast majority of divisions are observed. Although this hypothesis deserves further quantitative analysis, our observations and those of others (Hall et al., 1999) suggest that the length of DTC processes does not correlate with the zone border. This difference is especially evident in older adults: the zone size decreases, while processes stretch beyond the zone border (J.M. and E.J.A.H., unpublished results). We do not consider further the role of the DTC processes here.

Many additional general hypotheses exist for cell division behavior vis-à-vis DTC influence, all of which are consistent with the observation that cells exit mitosis and enter meiosis over a relatively small distance, presumably in response to a relatively sudden local change (e.g., threshold GLP-1 activity level). Our analysis refutes two of the most simple hypotheses for cell division behavior: (1) that all the cells within the mitotic zone have an equal probability of undergoing cell division as a function of distance from the DTC, and (2) that germ cell division frequency is directly correlated to ligand exposure, whereby a peak division frequency would occur among cells closest to the DTC. Other viable and not necessarily mutually exclusive scenarios exist whereby distal cells “read” an initial GLP-1 activity and respond with a set number of cell divisions (after which they enter meiosis), or whereby the position of peak division frequency reflects the time between which peak GLP-1 signaling activity occurs (distal-most) and cell division occurs as a response to it. Additional experiments are required to assess these and other hypotheses.

Several phenomena within the zone bear on the observed spatial pattern of cell division frequencies, including the relative speed with which cells move through the zone and relative cell cycle time. Indeed, these two factors are likely related since cells are presumably pushed in an overall proximal direction by cell divisions occurring distal to them, as well as by their own division. In addition, cells may be “pulled” proximally by vacancies created as a result of cell death or ovulation. Assuming that the dynamics of cell movement are similar within the distal half (1–10 CD) of the zone, our observation that cell division frequencies are reduced at the distal-most part of the zone is consistent with the hypothesis that the cell-cycle time of these distal-most cells is slower than that of their more proximal neighbors. Alternatively, the period of M phase of the cycle could occupy a shorter time of the total cell cycle in the distal-most cells, or cells could reside in the distal end for a

shorter time relative to the cells in the region of peak division frequency. Though our analysis cannot distinguish between these hypotheses, we favor the interpretation that the cell cycle is slower in the distal-most cells or that these cells spend more time in a quiescent state. This hypothesis, in turn, suggests that either very high levels of ligand, direct contact with the DTC, and/or an as-yet unknown distal factor negatively influence the frequency of distal germ cell division in the distal-most part of the zone. Regardless of the exact mechanism whereby the distal-most cells are observed to divide less frequently, the methods we have employed can be used for further comparison studies among different mutant strains and among worms in different life-stages to gain a greater understanding of comparative cell division behavior under these conditions.

C. elegans germ cells compared to other characterized adult stem cell systems

In several mammalian stem cell systems, including mammalian skin, intestine, hepatic, neural, and hematopoietic lineages (see Alison et al., 2004; Beites et al., 2005; Li and Xie, 2005; Marshak et al., 2001; Potten, 2004 for reviews), "transit amplifying" cells both divide and begin to express different markers after leaving the niche and prior to entry into a non-dividing differentiated state. A common (though not defining) feature of many adult mammalian stem cell systems is that the stem cells in the niche are slow-cycling or spend more time in a quiescent state relative to their transit amplifying cell progeny. Indeed, this feature has been exploited in the identification of stem cells and niches (e.g., Cotsarelis et al., 1990). One hypothetical scenario that is consistent with our observations is that the cell cycle of the distal-most germ cells in the *C. elegans* germ line may be slower relative to their dividing progeny, and would thus resemble stem cell/transit amplifying cell systems in mammals. Our data also indicate that rigorously quantitative measurements of relative cell cycle time will be necessary to test this hypothesis.

In many stem cell systems, multiple niche signals are integrated by stem cells, and cadherin-mediated interaction at the interface of the niche and stem cell is often critical for proper stem cell behavior (Li and Xie, 2005). Though there is not yet evidence for the involvement of these pathways in the *C. elegans* germ line, our observation that increasing GLP-1 activity did not increase the cell division rate of distal-most cells suggests that additional controls act on the distal-most germ cells.

If cell divisions within the *C. elegans* germline mitotic zone were strictly synchronous, we would expect to observe clusters of simultaneous divisions. In addition, we would expect a distal-to-proximal geometric increase in cell number within such clusters. While our analysis suggests that simultaneous divisions tend to cluster spatially, other than a dearth of divisions in the distal-most zone, we do not observe an obvious pattern of increasingly large clusters of synchronously dividing cells with respect to the distal proximal axis (based on examination of the 23 gonad arms that contained 7 or more divisions; data not shown). However, this type of pattern among cohorts of lineally related cells could easily escape detection because of slight asynchrony in division time and the relatively short proportion of the cell cycle that is detectable. Lineage analysis will be required to clarify this possibility. It will be of particular interest to determine if the cell divisions at the distal end are asymmetric, as they are in the *Drosophila* germ line.

A model for the dynamics of germline proliferation in C. elegans

Acknowledging that many possible scenarios of germ cell dynamics (integrating cell cycle and cell movement) are consistent with our observations, we speculate that germ cells in the *C. elegans* adult mitotic zone could behave as a stem cell/transit amplification system. In this model, germline stem cells would be slower-cycling cells in close contact with the DTC body, and daughter cells generated from stem cell divisions would leave the niche and produce lineally related cohorts of transit amplifying cells. These cells may be able to repopulate the niche and take on stem cell characteristics were the niche to be vacated, as has been demonstrated in *Drosophila* (Brawley and Matunis, 2004).

In our model, stem cell daughters would remain relatively close to each other as they undergo 1–3 loosely synchronous divisions. We speculate (see also Hansen et al., 2004b) that the switch to pre-meiotic S phase would occur when a cell in G1 experienced a critical loss of GLP-1 signaling and concomitant increase of meiosis-promoting activity (increased accumulation of GLD-1, for example; Hansen et al., 2004a). Some degree of cell-cycle asynchrony among the transit cells could account for the relatively smooth decline in cell division frequency over the proximal half of the zone in the adult.

Our model must also take into account the observation that, in earlier life stages, the mitotic zone comprises fewer cells, and the mitosis/meiosis border is both closer to the DTC and sharper (Hansen et al., 2004b). In addition, a change in germ cell sexual fate occurs in the hermaphrodite over the course of larval germline development (Ellis and Schedl, in press). Our preliminary observations suggest that the mitotic index in the L4 mitotic zone is significantly elevated over the adult and concur with earlier speculation that the sharper mitosis/meiosis border may result from more synchronous germ cell divisions that allow cells to reach the critical distance from the DTC in a more uniform stage of their cell cycle (Hansen et al., 2004a; J.M. and E.J.A.H., unpublished observations). The late-adult reduction in size of the mitotic zone (Killian and Hubbard, 2005) may result from a lengthening of the stem cell cycle and/or lengthening of the amplification division cell cycle or a reduction in the number of amplification divisions. Finally, many additional factors may influence cell proliferation frequency including nutritional status and rate at which cells exit the system by cell death or ovulation. Further comparative quantitative studies will be required to address these questions.

Notch receptors in mammals are implicated in many cancers. The results from a comparison of the *glp-1* mutant and wild-type intensity profiles are intriguing with regard to those cases

where Notch-mediated signaling promotes proliferation. If our speculation regarding a stem cell/transit amplifying cell model is correct, our data imply that the putative transit-amplifying cells are more profoundly affected by elevated GLP-1/Notch signaling than are the stem cells themselves. It will be of interest to determine if Notch signaling in mammalian stem cell systems affects the frequency of cell division of transit amplifying cells more than that of the stem cells themselves.

Our methods and results provide a rigorous foundation for future comparison studies of germline proliferation over the course of germline development, during reproductive senescence, and under additional life-history and mutant conditions that alter germline proliferation. More generally, these data offer insight into cell division behavior within an anatomically restricted population of dividing cells that do not appear to follow a uniform and invariant pattern of divisions. It is likely that many instances of localized cell proliferation are similar in this respect and that similar analytical approaches will be fruitful.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.12.046.

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